ISOLATION AND CHARACTERIZATION OF MUTANTS THAT ARE DEFECTIVE IN INACTIVATION OR DEGRADATION OF ASPARTATE TRANSCARBAMYLASE AND/OR GLUTAMINE PHOSPHORIBOSYLPYROPHOSPHATE AMIDOTRANSFERASE

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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

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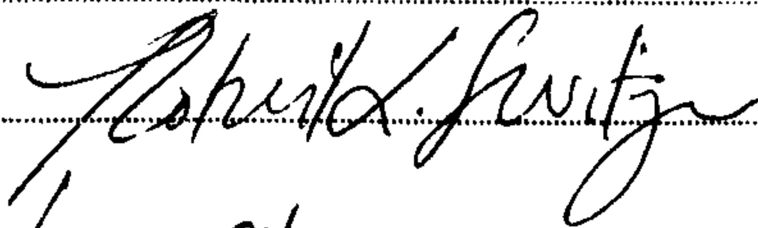

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TABLE OF CONTENTS

PAGE

INTRODUCTION 1

MATERIAL AND METHODS 4

I. BACTERIOLOGICAL PROCEDURE 4

A. BACTERIAL STRAINS 4

B. LIQUID AND PLATE MEDIA 4

C. CULTURE METHODS 7

D. HARVEST AND STORAGE OF CELLS 8

E. EXTRACT PREPARATION 9

II. NTG MUTAGENESIS 9

III. PHENOTYPIC EXPRESSION AND MUTANT ENRICHMENT CYCLE 10

IV. IMMUNOSCREENING 11

A. TRANSFER AND LYSIS 11

B. DNaseI TREATMENT 12

C. PROTEIN A - HRP CONJUGATE AND GOAT ANTI RABBIT - HRP CONJUGATE IMMUNO BLOTTING 12

D. ALKALINE PHOSPHATASE CONJUGATED GOAT ANTI RABBIT IMMUNOSCREENING SYSTEM 14

V. ENZYME ASSAYS 15

A. ASPARTATE TRANSCARBAMYLASE 15

B. GLUTAMINE PHOSPHORIBOSYLPYROPHOSPHATE AMIDOTRANSFERASE 16

C. PROTEIN ASSAY 17

VI. TRITIUM LABELING AND IMMUNOPRECIPITATE 17

VII. TUBE GEL ELECTROPHORESIS 18

VIII. MATERIALS 21

RESULTS 22

DISCUSSION 47

BIBLIOGRAPHY 52

INTRODUCTION

This thesis forms part of a larger study examining the biochemistry and regulation of the degradation-inactivation of two enzymes involved in nucleotide biosynthesis - aspartate transcarbamylase and glutamine phosphoribosylpyrophosphate amidotransferase. Aspartate transcarbamylase (ATCase), which catalyzes the second step of pyrimidine nucleotide biosynthesis, and glutamine phosphoribosylpyrophosphate amidotransferase (ATase), the first enzyme in the purine nucleotide biosynthetic pathway, are rapidly inactivated and undergo energy-dependent degradation when cells are starved for nutritional requirements, such as glucose, nitrogen, or amino acids (Turnbough and Switzer, 1975, Deutscher and Kornberg, 1968, and Maurizi et al., 1978). The regulation of inactivation and degradation of these two enzymes are evidently under control of a very complex system.

The level of ATCase in cells growing exponentially on NH_4^+ as the sole nitrogen source is maintained stable by simultaneous synthesis and degradation (Bond et al., 1983). However, under other growth conditions ATCase is not degraded during exponential growth. As cells enter stationary growth where inactivation begins, synthesis of ATCase is stopped (Maurizi and Switzer, 1978). The intracellular level of pyrimidine nucleotides controls the rate of ATCase synthesis but has little effect on degradation rate (Bond et al., 1983). Bond et al. (1983) also showed that the presence of various amino acid have an impact on degradation rate. When aspartate, glutamine, isoleucine,

proline, and threonine are added to the growth medium, degradation of ATCase is suppressed to an undetectable rate (Bond *et al.*, 1983). Under all conditions tested, degradation and inactivation of ATCase appear to be simultaneous events. So far, attempts to block inactivation without inhibiting degradation have not been successful (Maurizi *et al.*,1978; Brabson and Switzer, 1975)

It is sensible to regulate ATCase and ATase in some parallel manner since similar amounts of pyrimidine and purine nucleotides are required for cell growth. Both enzymes undergo inactivation and degradation under the same conditions, but the regulation of these processes may not be the same. In contrast to ATCase, ATase is stable in cells growing exponentially on NH_4^+ as the sole nitrogen source. Studies have shown that ATase undergoes an oxygen-dependent inactivation (Bernlohr and Switzer, 1981), which is followed by degradation (Ruppen and Switzer, 1983). Apparently, inactivation of ATase is due to oxidation of a (4Fe-4S) cluster, which serves as a prosthetic group for this enzyme (Bernlohr and Switzer, 1981). Inactive cross-reactive protein can be generated by addition of chloramphenicol or rifampin prior to the onset of degradation (Ruppen and Switzer, 1983). The energy dependence of ATase degradation is less easily detected than that of ATCase.

Evidence shows that the stringent response is involved in regulating inactivation and degradation of both enzymes, but the regulation is not mediated by (p)ppGpp (Bond and Switzer, 1984).

In the case of ATCase, degradation is defective in *rel* mutants (Bond and Switzer, 1984). The degradation rate of ATCase is reduced in *relA* mutants, except upon glucose starvation, and is undetectable in *relC* mutants upon amino acid starvation. Similarily, ATase inactivation in *rel* mutants is slower than in wild type strains (Bond and Switzer, 1984). As a matter of fact, ATase degradation is almost totally blocked in *relC* mutants.

The purpose of this thesis project is to isolate and characterize mutants that are defective in inactivation or degradation of ATCase and/or ATase. The approach to enriching and screening for such mutants was devised by Drs R. L. Switzer and L. B. Bussey. On the basis of work by Smits *et al*. (1984), they reasoned that cells that fail to inactivate and/or degrade biosynthetic enzymes during starvation would grow out faster after a period of starvation than wild type cells. After many cycles of repeated selection for the cells that grow out most rapidly, the surviving colonies were screened for those that retained elevated levels of ATCase and/or ATase using an immunochemical staining method which was developed during these studies. Hopefully, genetic and biochemical analyses of these mutants can lead us to define the biochemical components and mechanisms of the inactivation-degradation processes.

MATERIALS AND METHODS

I) Bacteriological Procedures

A) Bacterial Strains

The parental strain of Bacillus subtilis used in these studies was DB104 (his, nprR2, nprE18, aprA3), a histidine auxotroph and double mutant deficient in the extracellular alkaline and neutral protease, obtained from Roy H. Doi (1984). Other B. subtilis and Escherichia coli strains were also used as positive and negative controls. See Table 1 for detailed genotypes.

B) Liquid and Plate Media

BMM and VBG were used as minimal media. SNB and MLB were used as rich media. To prepare agar plates, 20 grams of Difco agar was added per 1 of liquid medium. All additives were added after the medium was autoclaved and allowed to cool.

1) BMM Minimal Medium (Anagnostopoulos and Spizizen, 1961)

The following stock solutions were prepared and sterilized separately.

i) 25X K-phosphate stock solution

anhydrous KH_2PO_4	350 g/1

ii) 25X BMM salts solution

$(NH_4)_2SO_4$	50 g/1
$MgSO_4 \cdot 7H_2O$	12.5 g/1
Na_3-citrate$\cdot 2H_2O$	25 g/1

TABLE 1. POSITIVE AND NEGATIVE CONTROL STRAINS

STRAIN	BACTERIAL SPECIES	GENOTYPE
EB47	E. coli	hisG1, purF1, argH1, thi-1, pyrB59, ara-13, lacY1, or lacZ4, malA1, xyl-7, mtl-2, rpsl8,9 or 14, tonA2 or tsx-25, λR⁻, λ⁻, supE44, hsdR4, recA56/pCB101*
EB48	E. coli	hisG1, purF1, argH1, thi-1, purB59, ara-13, lacY1, or lacZ4, malA1, xyl-7, mtl-2, rpsl8,9 or 14, tonA2 or tsx-25, λR⁻, λ⁻, supE44, hsdR4, recA56/pCB201**
TB2	E. coli	Δ argI-pyrBI/pLS210#
TX158	E. coli	ara, lac, [purF200·lac::pl(209)] /pPZ2^
BR16	B. subtilis	trpC2, lys

* pCB101 = pGB2/B. subtilis purB
** pCB201 = pGB2/B. subtilis purF
\# pLS210 = pUC13/B.subtilis pyrB
^ pPZ2 = pBR322/B. subtilis purF

Ref:
EB47 and EB48 were constructed by Dr. L. B. Bussey in this laboratoy.
BR16 : Swanton and Edlin, 1972
TB2 : Hoover et al., 1983
TX158: Smith and Gots, 1980
pLS210 : Lerner and Switzer, J. Biol. Chem. 1986 in press.
pPZ2 : Makaroff et al., 1983

iii) 500X SNB salts solution

$CaCl_2 \cdot 2H_2O$	3.68 g/l
$FeSO_4 \cdot 7H_2O$	7 mg/l
$MnCl_2 \cdot 2H_2O$	100 mg/l
KCl	25 g/l
$MgSO_4 \cdot 7H_2O$	6.26 g/l

To prepare 1 l of BMM liquid medium, 760 ml of distilled water was added to a 2 l flask and 40 ml of 25X K-phosphate stock solution was added to a 500 ml flask containing 160 ml of distilled water. Both flasks were autoclaved and allowed to cool. Then the following was added to the 2 l flask: the diluted K-phosphate solution, 40 ml of sterile 25X BMM salts solution and 2 ml of 500X SNB salts solution.

2) VBG Minimal Medium (Vogel and Bonner, 1965)

The 50X VBG solution was prepared as following:

H_2O	670 ml
$MgSO_4 \cdot 7H_2O$	10 g
Citric acid H_2O	500 g
$Na(NH_4)HPO_4 \cdot 4H_2O$	175 g

To prepare 1 l of VBG, 20 ml of 50X VBG solution was autoclaved with 980 ml of distilled water in a 2 l flask.

3) SNB Rich Medium

SNB rich medium was made up of 0.8% Difco nutrient broth plus SNB salt solution (40 ml to 1 l of medium).

4) MLB Rich Medium

The following were added to one liter of distilled water.

Bacto-Tryptone	10 g
Bacto-Yeast Extract	5 g
NaCl	5 g
Glucose	1 g
Tris 1M (pH 7.5)	10 ml

This solution was autoclaved. After cooling 1 ml of 1M $MgSO_4 \cdot 7H_2O$ was added to the solution.

5) Other Additions

Stock concentrations		Medium concentration
Glucose	40%	0.4% or 0.1%
Histidine	15 mg/ml	50 ug/ml
Arginine	15 mg/ml	50 ug/ml
Adenine	15 mg/ml	50 ug/ml
Guanosine	15 mg/ml	50 ug/ml
Uracil	2 mg/ml	20 ug/ml
Thiamine	5 mg/ml	5 ug/ml

C) Culture Methods

All strains were stored as dimethylsulfoxide (DMSO) permanent cultures at -80°C (Revco ultra low freezer). To prepare a DMSO permanent culture, 1.5 ml of a overnight rich culture was added to a previously autoclaved 2 ml-glass vial (1/2 dram, screw cap Kimble #60910-C) containing two drops of DMSO.

For standard growth experiments, 30 ul of a DMSO permanent culture was added to 5 ml SNB and allowed to grow for 8 to 10 hours or overnight at 37°C with shaking (300 RPM) (New Brunswick Scientific controlled environment incubator shaker). One ml of this culture was transferred to a 500 ml flask containing 100 ml of previously warmed minimal medium (0.4% glucose). This culture was incubated with shaking at 300 RPM, 37°C overnight. This overnight minimal culture was then diluted into 1 l of fresh minimal medium (0.4% glucose or 0.1% glucose for glucose starvation experiments) to give approximately 10 Klett Units. Growth was followed by measuring the turbidity of the culture fluid in a Klett-Summerson colorimeter with a red No. 66 filter. Cultures were routinely plated to check for His^{+} contaminants; none were found.

D) Harvest and Storage of Cells

One hundred to two hundred and fifty ml of culture were withdrawn at given times and centrifuged in a Sovall RC2B at 16,000 x g for 5 min at 4°C (GSA rotor). The pellets were washed once with buffer A (50 mM Tris/HCl, pH 7.9, 10 mM $MgCl_2$ and 0.1 mM EDTA, at 4°C) and centrifuged again at 12,000 x g for 10 min at 4°C in a SS34 rotor. The washed pellets were quickly frozen with liquid nitrogen and stored at -20°C.

E) Extract Preparation

Cell pellets were resupended (30 times the concentration in the medium from which they were harvested) by adding the appropriate amount of cold Buffer A, containing 10 mM B-mercaptoethanol and 1 mM phenylmethylsulfonylfluoride. The cells were broken by sonication on ice in a Branson probe-type sonicator (Model W185, 1/2" probe). Sonication was done in 20 (or occasionally 15) sec bursts (at setting 7, approximately 75 watts), with 1 min cooling intervals between bursts to prevent overheating. The actual sonication time varied from 1 min to 2 min (depending on the volume of cells harvested). Cellular debris was removed by centrifugation for 15 min at 12,000 x *g* at 4°C in a SS34 rotor.

II) N-methyl-N-nitro-N-nitrosoguanidine (NTG) Mutagenesis (Adelberg *et al*., 1965; Uehara *et al*., 1979)

One ml of a DB104 SNB starter culture was inoculated into 50 ml of SNB medium in a 500 ml sidearm flask. This 50 ml culture was grown to 90 Klett Units at 37°C. Twenty ml was removed and centrifuged in a SS34 rotor at room temperature (12,000 x *g*) for 5 min. The suppernatant was discarded. The pellet was washed once with 20 ml of TM buffer (50 mM Tris-malate, pH 6.0 at 37°C). Cells were then resuspended in 20 ml of the TM buffer, and 400 ul of 5 mg/ml NTG in sterile water was added to give a final NTG concentration of 100 ug/ml. These cells were incubated in a 37°C shaker at 300 RPM. A 5 ml aliquot

was removed every 15 min for one hour. The cells were collected by vacuum filtration onto a nitrocellulose membrane (0.45 um pore size, 25 mm diameter) using 15 ml Millipore glass filter holders, fitted with 250 ml vacuum filtering flasks. The collected cells were washed twice with 5 ml of TM buffer. The filters were placed into test tubes containing 8 ml of SNB medium and mixed rapidly with a Vortex mixer to remove the mutagenized cells. Two ml of glycerol was added to the cell suspensions. These glycerol-cell suspensions were then transferred to screw cap centrifuge tubes, quick frozen in liquid N_2, and stored at -80°C.

III) Phenotypic Expression and Mutant Enrichment Cycle

Phenotypic expression was performed by inoculating 1 ml aliquots of the NTG treated cells into 50 ml of BMM medium containing 0.4% glucose, and 50 ug/ml histidine in 500 ml flasks. These cultures were grown until mid-log phase and used as inoculum for mutant enrichment. The enrichment procedure was a modification of Smits *et al*. (1984). The following enrichment cycle was repeated 12 times for each phenotypically expressed culture: 10 ml of the mid-log culture was added to 90 ml of previously warmed BMM medium containing 0.1% glucose, and 50 ug/ml histidine. These cultures were allowed to enter stationary phase and starve (for glucose) for approximately 6 hours before 10 ml was transferred to previously warmed 500 ml flasks containing 90 ml of BMM medium containing 0.1% glucose, and 50 ug/ml histidine. At the end of enrichment cycle, glycerol was

added to each culture to a final concentration of 20% by vol. These cells were then transferred to sterile 1.5 ml Microfuge tubes, quick frozen in liquid N_2 and stored at -80°C. Appropriate dilutions were spread on BMM plates (plus and minus histidine) and SNB to check for auxotrophs and His^+ revertants. No His^+ revertants were detected.

IV) Immunoscreening

A) Transfer and Lysis

The NTG treated and enriched cells (appropriate dilutions, 100 to 200 colonies per plate) were spread on BMM plates containing 0.4% glucose, and 50 ug/ml histidine, incubated at 37°C in a Blue M incubator until reasonable size (1 or 2 mm diameter) colonies were formed (usually 36 to 48 hours). Two replicate plates were made from each master plate. Colonies from the master plate were transferred to a Whatman #1 filter and carefully used to inoculate two new plates. The replication apparatus consisted of a wooden block (8.2 cm in diameter) covered with three sheets of Kleenex tissue and a 15 cm diameter Whatman #1 filter. The filter was held in place by a large hose clamp. The replica plates were incubated for another 36 to 48 hours at 37°C. One of the replica plates was stored at 4°C to maintain viable cells. The other replica plate was used for lifting colonies. A properly labeled 8.6 cm diameter filter (Whatman #40 or nitrocellulose) was placed on the agar plate until moisture soaked through. This filter was then transferred

to a 100 mm glass petri dish, containing five blotters of Whatman # 1 filter disks (9 cm in diameter) saturated with fresh lysis buffer (0.1 M sodium bicarbonate, 1% Tween-20 and 2 mg/ml lysozyme). This petri dish was then incubated in an atmosphere of $CHCl_3$ in a large vaccuum dessicator for 30 min. The filter was transferred with the blotters to -20^oC for 20 min or until ready for DNaseI treatment.

B) DNaseI treatment

The lysozme treated filter was thawed at room temperature, transferred to a 1 l beaker containing 10 ml of DNaseI solution (10 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 100 mM NaCl, 0.1 mg/ml gelatin, and 2 ug/ml DNase I), and incubated for 1 hour with gentle shaking (Eberbach reciprocal shaker). The filter was then washed with 50 ml of buffered blocking solution (20 mM Tris/HCl, pH 7.5, 500 mM NaCl and 3 % gelatin) for 30 min, then twice with 50 ml of TTBS (20 mM Tris/HCl, pH 7.5, 500 mM NaCl and 0.05% Tween 20) for 5 min each time. The washes were performed at room temperature with gentle shaking. At this stage, the filter was ready for antibody treatments and color development.

C) Protein A - Horseradish Peroxidase Conjugate (HRP) and Goat Anti-Rabbit-HRP Conjugate Immuno Blotting

These immunoblotting systems were obtained from Bio-Rad and the manufacturer's protocol was followed as provided with slight modifications. The positive colonies produced purple colored spots on the filters.

Reagents:

1. Tris-buffered saline (TBS):

   20 mM Tris/HCl, pH 7.5, 500 mM NaCl

2. Tween-20 wash solution (TTBS):

   20 mM Tris/HCl, pH 7.5, 500 mM NaCl, 0.05% Tween-20

3. Antibody buffer:

   1% gelatin-TTBS

4. HRP color development solution:

   a. Sixty mg HRP color development reagent (4-chloro-1-napthpol) was dissolved in 20 ml ice cold methanol. The HRP color development reagent was made fresh daily.

   b. Immediately prior to use, 60 ul of ice cold 30% H_2O_2 was added to 100 ml room temperature TBS. This solution was mixed with the HRP color development reagent.

Procedure:

The DNaseI treated filter was placed colony side down in a 100 mm Petri dish containing 2 ml of diluted primary antibody. Rabbit anti-ATCase (Bond et al., 1983) and rabbit anti-ATase (Ruppen and Switzer, 1983) were used as primary antibodies. The dilutions were 1:500 for anti-ATCase and 1:1000 for anti-ATase. After 1 hour of incubation the filter was washed twice with 0 ml of TTBS for 5 min each wash. Then the filter was incubated with Protein A-HRP conjugate solution or goat anti-rabbit IgG (H+L) HRP conjugate solution 1:1000 dilution for both) for 1 hour at room temperature. The TTBS washing step was repeated, and an

addtional TBS wash was followed. The color development reaction was started by immersing the filter into 15 ml of color development solution for 15 to 30 min. To stop the color reaction, the color development solution was washed off with distilled water.

C) Alkaline Phosphatase Conjugated Goat Anti Rabbit Immunoscreening System

This immunoscreening system kit was otained from Promega and the manufacturer's protocol was followed as provided with slight modifications. The positive colonies produced dark purple spots on the filters.

Reagents:

1) Tris buffered saline + Tween 20 (TBST):

   10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20

2) Alkaline phosphatase (AP) buffer:

   100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$

3) Reaction stop/storage buffer:

   20 mM Tris/HCl, pH 8.0, 5 mM EDTA

4) Color development substrate solution:

   5 ml AP buffer + 33 ul nitrobluetetrazolium (NBT) substrate + 16.5 ul 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate. The solution was mixed well and protected from light. It was made immediately prior to use.

Procedure:

The DNaseI treated filter was transferred into a 100 mm glass petri dish containing 7.5 ml of diluted primary antibodies (1:1000 for anti-ATCase and 1:2000 for anti-ATase, in TBST solution). After 30 min incubation, the filter was washed 3 times with 20 ml of TBST solution for 5 min each wash. Then, the filter was incubated with 7.5 ml of diluted anti-rabbit IgG alkaline phophatase conjugate (1:7500 dilution in TBST) for 30 min. The TBST washing steps were repeated. The excess moisture on the filter was removed by placing it on top of dry filter paper. Then, the filter was incubated with 1 ml of color development substrate solution. The color reaction was stopped by immersing the filter in stop/storage solution when desired color intensity was obtained.

Note: All the diluted antibody solutions and the diluted anti-rabbit IgG alkaline phosphatase solution were reusable (at least 3 times).

V) Enzyme Assays

A) Aspartate Transcarbamylase (Bond _et_ _al_., 1983)

Aspartate transcarbamylase was assayed in 200 mM Tris-acetate, pH 8.2, 50 mM L-aspartate, 10 mM carbamyl phophate, 25 ul to 100 ul of cell extract, and water in a final volume of 1.0 ml. This reaction was started by the addition of cell extract. The assay mixture was incubated at 30°C for 20 min. The reaction was stopped by adding 1.0 ml of 5% perchloric acid, and cooled on

ice for 20 min. The tubes were centrifuged at 80% of full speed for 5 min in a clinical centrifuge to remove preciptated protein. Then 0.1 ml of each supernatant was transferred to a new tube, containing 0.9 ml of water, 0.5 ml of 0.5% diacetylmonoxime in 5% acetic acid and 1.0 ml of a solution of 5 g/l of antipyrine in 50% sulfuric acid in water. This reaction mixture was mixed vigorously and incubated in a 60°C water bath for 2 hours under yellow light along with standards of known carbamyl aspartate concentration. The color formed was measured at 466 nm on a Beckman Acta CIII spectrophtometer.

B) Glutamine Phosphoribosylpyrophosphate Amidotransferase

Amidotransferase activity (Meyer and Switzer, 1979) was measured in a two-step spectrophotometric glutamate production assay. In the first step, 0.1 ml of cell extract was added to 0.9 ml of primary reaction mixture, containing 50 mM Tris/HCl, pH 8.2, 2 mM EGTA, 5 mM $MgCl_2$, 2.5 mM phosphoribosylpyrophosphate (PRPP), and 20 mM glutamine. An identical duplicate except excluding PRPP was used as a blank for general glutaminase activity. These two sets of tubes were incubated at 37°C for 20 min. The reaction was stopped by heating at 100°C for 2 min, then cooled on ice. The tubes were centifuged for 5 min in a clinical centrifuge. Then, 100 ul of the supernatant was transferred from the first step to the second step reaction mixture which contained 100 mM potassium phosphate, pH 7.9, 0.6 mM acetylpyridine adenine dinucleotide and 1.5 ul of glutamate

dehydrogenase (135 mg protein/ml), incubated at 37°C for 45 min. The absorption at 363 nm was read on a Beckman Acta CIII spectrophotometer.

C) Protein Assay

Protein concentration was determined by the method of Lowry *et al*. (1951) using crystalline bovine serum albumin (BSA) as the standard protein. The cell extract was diluted in Buffer A to appropriate dilutions (usually 1:15 and 1:30), and 0.1 ml of each dilution was added to 1 ml of reaction mix (2% Na_2CO_3 in 0.1 N NaOH, 0.1% $CuSO_4 \cdot 5H_2O$, 0.2% Na-tartrate). Then the mixture was gently shaken by hand. After 10 min at room temp, 50 ul of 2 N phenol reagent was added and immediately mixed on a Vortex mixer. Then the tubes were incubated at room temp for 15 min. The absorbance at 750 nm was measured with a spectrophotometer.

VI) Tritium Labeling and Immunoprecipitate

For [^{3}H]leucine labeling, cells were cultured as per glucose starvation experiments. When the 1 l culture reached turbidity reading of 30 KU, 1 ml of 1 mCi/ml of [^{3}H]leucine (56 Ci/nmole) was added to the cell culture. At 100 KU, 150 ml of cell culture was harvested. At 120 KU (presumed to be T_0), 13.33 ml of 15 mg/ml of nonradioctive leucine was added the cell culture. Then 150 ml aliquots were harvested every hour for 4 hours.

The cells were sonicated as usueal, but instead of spinning 15 min at 12,000 X *g* in a SS34 rotor, the cell debris was removed

by spinning at 35,000 RPM at 4°C in an AH650 rotor for 30 min in a Sorvall OTD65B ultracentrifuge.

The enzyme activities of ATCase and ATase were determined for each sample. Then the amount of each enzyme present in 1 ml of extract was calculated. One ug of ATCase has a specific activity of 0.5 nmole/min/ml (Brabson and Switzer, 1975) and 1 ug of ATase, 40 nmole/min/ml (Wong et al., 1981). In order to get a conveniently workable size of pellet, pure non-radioctive enzymes were added to a final concentration of 20 ug. To immunoprecipitate ATCase from one ml of extract, 45 ul of anti-ATCase was added to each sample. Sixty ul of anti-ATase was used to precipatate ATase. Immunoprecipitation tubes were placed at 4°C overnight and then spun for 2 min in a Microcentrifuge at 4°C. The supernant fluid was carefully removed, and the pellets were washed 3 three time with 1 ml wash buffer (50 mM Tris/HCl, pH7.9, 1 M NaCl, 0.1 mM EDTA, and 1% Triton X-100). The pellets were then washed with 1 ml of H_2O and frozen at -80°C.

VII) Tube Gel Electrophoresis

Reagents:

1. running gel stock:

   30 g acrylamide, and 0.8 g bis-acrylamide to 100 ml with H_2O and filtered with Millipore filter unit, 0.45 um filter.

2. running gel buffer stock:

   36.3 g Tris/HCl, pH 8.8, 0.84 ml TEMED, and 0.8 g SDS to

200 ml with H_2O. This solution was kept cold.

3. stacking gel stock:

   12.0 g acrylamide, and 0.32 g bis-acrylamide to 100 ml with H_2O and filter with Millipore filter unit, 0.45 um filter.

4. stacking gel stock buffer:

   4.84 g Tris/HCl, pH 6.8, 0.32 g SDS to 200 ml with H_2O and was kept cold

5. 10X chamber buffer stock:

   144 g glycine, and 30 g Tris and added water to 1 l

6. 2X sample buffer:

   0.788 g Tris/HCl, pH 6.8, 2.0 g SDS, 10 ml glycerol added water to 50 ml

7. Immuno sample buffer:

   2 ml 2X sample buffer, 200 ul B-mercaptoethanol, 20 ul bromphenol blue (saturated solution in water)

8. Scintillation fliud:

   946 ml toluene, 4 g 2,5-diphenyloxazole (PPO), and 54 ml undiluted tissue solubizer (0.5N) (obtained from Research Product International Crop.)

Procedure:

Glass tubes were soaked in Photoflo solution (1:200 dilution), air dried and then put in a casting stand. With a 1 ml Pipetman 2 ml of running gel was added to each tube. Then 250 ul of H_2O was carefully layered over the gel, and the gels were

allowed to polymerize for 30 min. The running gel consisted of the following: 8 ml of running gel stock, 6 ml running gel buffer stock, 3 ml fresh ammonium persulfate solution (2 mg/ml), and 7 ml of H_2O. When the running gels had polymerized completely, the overlaying water was poured off. Then 200 ul of stacking gel was added per tube and allowed to polymerize for 30 min. The stacking gel was prepared as follows: 1 ml stacking gel stock, 2.5 stacking gel buffer stock, 0.5 ml fresh ammonium persulfate solution (2 mg/ml) and 12 ul TEMED. During stacking gel polymerization 1 l of chamber buffer (100 ml of 10X chamber buffer stock solution, 10 ml of 10% SDS and 890 ml of water) was prepared. The immunoprecipitated pellets were thawed with 100 ul of immuno sample buffer. Then each sample was mixed on a Vortex mixer for 10 seconds, boiled for 10 min and mixed again on a Vortex mixer for 10 sec. The samples were allowed to cool before being loaded onto tube gels. Each sample was carefully loaded with a 200 ul Pipetman onto a tube gel, which had been placed in a gel box filled with chamber buffer. The samples were run through the stacking gels at 1.5 mamp/gel (constant current). When the samples had run into running gel, the current was increased to 3.0 mamp/gel. The current was turned off when the tracking dye was about 1 mm from the bottom of the gel. The total running time was approximately 3 hours. The tubes were removed from gel box one at a time. The gel was removed from the tube by use of a long 20 gauge needle and syringe filled with water. The needle was inserted from the stacker side and the gel

was carefully reamed at the edge of the tube. The gel was easily blown out of the tube by using a Pasteur pipet bulb. Then the gel was sliced with a gel slicer (Bio Rad model 165-2500) into 1 mm thick disks. Each slice was transferred to a plastic 5 ml minivial. Five ml of scitillation fluid was added to each vial which was then capped, labeled and incubated at 50°C for 2 hours. At the end off incubation, each vial was Vortexed and allowed to cool before placing in a Beckman (model LS 7000) scintillation counter. Each vial was counted for two min.

VIII) Materials

Most reagents were reagent grade and obtained from J. T. Baker Chemical Co. or Sigma Chemical Co. except as specified otherwise.

RESULTS

1) Mutagenesis and Enrichment Cycles

NTG is a very powerful mutagen which can introduce multiple mutations and rare mutations that can not be obtained by other means. The longer the B. subtilis cells are treated with NTG, the higher the mutation rate. The mutation rate can be estimated by the frequency of auxotrophic mutants. The results of counting auxotrophs for each NTG treated DB104 cell culture is consistant with this statement. The frequency of obtaining auxotrophs from 15 min, 30 min, 45 min, and 60 min NTG treatment of cell cultures is presented in Table 2. It should be noted that the auxotrophic rate after 45 min of NTG treatment was extremely high (over 81%). This may reflect the fact that only a small number of cells survived extended treatment. Although a higher mutation rate is obtained from longer NTG treatment, it is not beneficial to treat cells for extensive periods of time. This is because NTG not only mutates but also kills cells at very high frequency (see Table 3). The killing levels were greater than 99% after 15 minutes of treatment with NTG.

Phenotypic expression is an important procedure following mutagenesis. During phenotypic expression no selective pressure for or against the mutant of interest is made; it is a period of normal cell growth. Induction of chromosomal mutations by NTG is most efficient when the mutagen is added to exponentially growing cells in which DNA replication and protein synthesis are very rapid. Exponentially growing cells have multiple copies of

Table 2. Frequency of Auxotroph Isolated After NTG Treatment of *B. subtilis* DB104 Cells

Time of treatment	Colonies on SNB	Colonies on BMM+his	Dilution of cells	% of* auxotrophs
15 min	100	95	10^4	5%
30 min	135	119	10^3	12%
45 min	76	15	5×10^2	81%
60 min	50	4	5×10^2	92%

$$* \quad \% \text{ of auxotrophs} = \frac{(\#\text{ colonies on SNB} - \#\text{ colonies on BMM+his})}{\#\text{ colonies on SNB}} \times 100$$

Table 3. Survial Rate After NTG Mutagenesis of *B. subtilis* DB104 Cells

Incubation time with NTG	Colonies per ml *	Survival rate
0 min	7.2×10^{8}	100%
15 min	1.0×10^{6}	1.4×10^{-1}%
30 min	1.4×10^{5}	1.9×10^{-2}%
45 min	3.6×10^{4}	5.0×10^{-3}%
60 min	1.4×10^{4}	1.9×10^{-3}%

* This was determined by plating 1:10 serial dilution on SNB agar plates, counting the number of colonies and multplying by the dilution.

$$\text{Survival rate equals } \frac{\text{\# of colonies per ml}}{\text{\# of colonies per ml at 0 min}} \times 100$$

chromosomal DNA and a high titer of cellular proteins. Phenotypic expression allows segregation of chromosomal DNAs and allows the mutated DNAs to be transcribed and translated. During phenotypic expression, proteins produced before mutagenesis are diluted out by cell division and protein turnover.

The enrichment cycles were performed to increase the concentration of mutants that did not degrade ATCase and/or ATase during 6 hours of glucose starvation. The half lives of ATCase and ATase under glucose starvation are 90 min in wild type _B. subtilis_. Therefore, after 6 hours of glucose starvation the level of both these enzymes in wild type cells should be about 6.25% of the original concentration. Presumably, the mutants of interest would resume exponential growth more rapidly than wild type cells when transferred to fresh medium lacking purines and pyrimidines. Thus, the cells that normally degrade ATCase or ATase would be diluted out eventually. As the enrichment cycles proceeded, the lag time prior to growth for each culture decreased as predicted (from 5 to 6 hours to about 2.5 hours). The enrichment cycles also helped to remove auxotrophs. Since hisitidine was the only amino acid provided and no vitamins or nucleotides were added, auxotrophic mutants other than His$^-$ could not grow.

2) Immunoscreening

The mutated cells gave rise to colonies that were different in size and appearence. Some colonies were larger than others.

Some colonies were smooth whereas others were rough. The smooth colonies stuck to the filters better than the rough ones. In general, cells stuck to nitrocellulose filters better than to Whatman #40 filters, which eventually were not used in immunoscreening experiments because they also gave very high color background.

The HRP immunoblotting kits that were obtained from Bio-Rad were less sensitive and less stable than the AP immunoblotting system. The antibody concentrations suggested by Bio-Rad were 1:10,000 dilution but the actual concentrations required were 1:500 dilution (anti-ATCase) and 1:1,000 dilution (anti-ATase and secondary antibody). Moreover, the color formation was very light sensitive and unstable. If the filter was exposed to light after color formation, the color faded away very rapidly. Sometimes the color faded away during colony picking. On the other hand, the AP immunoblotting system, which was obtained from Promega, was more sensitive, and the color formation was very stable. For primary antibody binding a 1:1,000 dilution for anti-ATCase and 1:2,000 dilution for anti-ATase were used. The concentration of secondary antibody suggested by the manufacturer, 1:7,500 dilution, worked well. Another advantage of using the AP immunoblotting system was that all the diluted antibody solutions were reusable (at least three times). Other advantages that favored the AP immunoblotting system were stable color formation and the ability to increase color intensity by wetting the filter.

The colonies that showed up as solid purple spots (Figure 1 and 2) after color development were presummed to be mutants that did not degrade either ATCase or ATase (depending on the primary antibody used). This was in contrast to purple circles or "halos" (Figure 3) that were produced from the parental strain DB104. Also notice that colonies on each plate did not produce uniform intensity. This may result from colonies that were at different stages of growth or from differing efficiencies of colony lifting and lysis. Under all conditions tested ATCase always produced weaker color than ATase. The positive colonies were picked and purified by restreaking three times before they were retested with both anti-ATCase and anti-ATase. Ten different strains that produced dark color against ATCase and/or against ATase were selected for further charaterization.

3) Characterizations of Mutants

The ten strains, named SW1 to SW10, were grown on BMM medium containing 0.1% glucose and 50 ug/ml of histidine and harvested at 2.5 hours after entering stationary growth. Then the specific activities for ATCase and ATase were determined for each strain. The results of this experiment and of AP immunoblotting are presented in Table 4. The purpose of this glucose starvation was to help define mutants for further characterization. After 2.5 hours of glucose starvation, the levels of both enzymes should be less than 40% of that at T_0, if the enzymes were being inactivated at normal rate (90 min half life). Using cells harvested

Figure 1. AP Immunoblotting Filters of NTG Mutated Cells

These are NTG mutated cells plated after 12 enrichment cycles onto BMM, 0.4% glucose and histidine agar plates. The filter on the left was screened for ATase, and the filter on the right was screened for ATCase. Colonies that produced solid dark dots were picked and purified for charaterization.

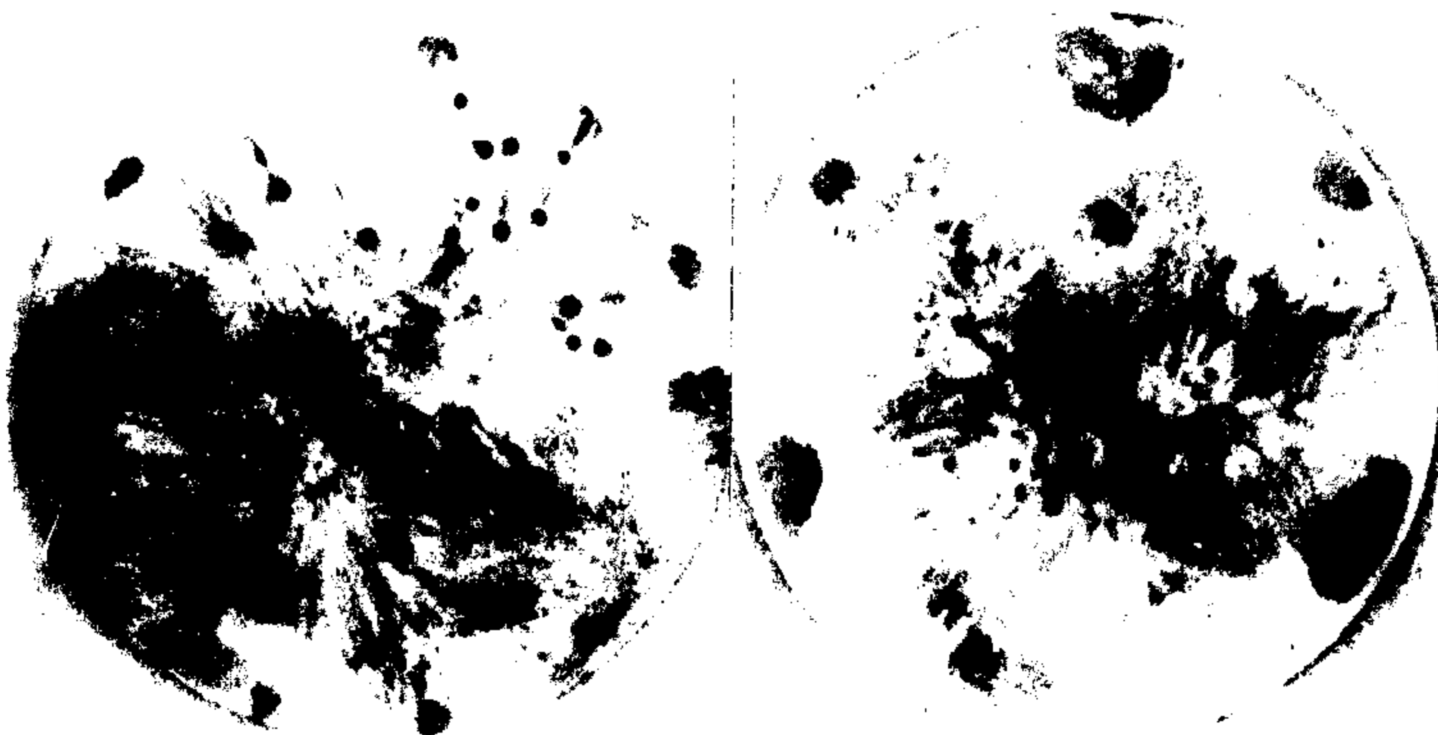

Figure 2. AP Immunoblotting Filter of Strain SW9

For demonstration, SW9 was plated on BMM, 0.4% glucose, and histidine agar plates and retested with anti-ATase (left) and anti-ATCase (right).

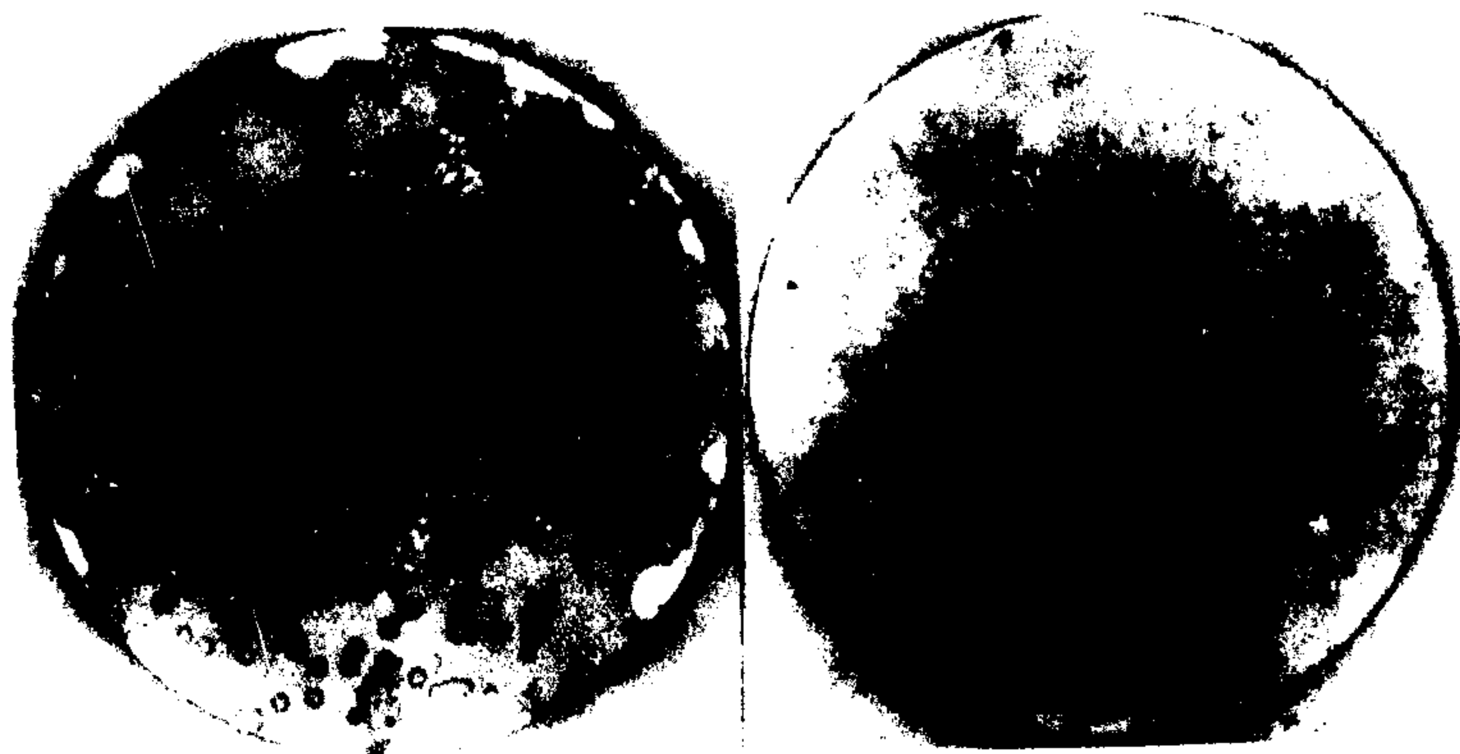

Figure 3. AP Immunoblotting Filters of Strain DB104

The filter on the left was screened for ATase and the one on the right, for ATCase. On both filters, colonies produced purple circles. Also note that under all conditions tested, ATCase always produced weaker color than ATase.

at this time we could distinguish mutants that had different inactivation rates by comparing the level of enzyme activities. If the half life was shortened, the enzyme activity would be at a very low or undetectable level. On the other hand, the enzyme activity would be a higher (compared to DB104), if the enzymes were inactivated at a slower rate. Strain SW4 (strongly positive with anti-ATCase) is not listed in Table 4 because the sample was lost during sonication. Several samples had high glutaminase activity, so that the ATase activity was undectable. Based on the levels of enzyme activity and immunologically cross reactive material (CRM), these mutants can be classified into at least 5 groups. The first group consists of SW9 and SW10, which have high levels of enzyme activity and CRM for both ATCase and ATase. The level of ATCase in SW10 may reflect constitutive expression. The second group consists of SW3, SW4, and SW5, which have a high level of enzyme activity and CRM for ATCase but little or no activity for ATase and various levels of CRM for ATase. The third group includes SW1, SW2, and SW7, which have various levels of CRM for both ATCase and ATase but very low or no activity for both enzymes. The fourth group is made up of SW8, which has no activity but moderate level of CRM for ATCase and high activity but low CRM for ATase. SW6 belongs to the fifth group which has high activity and CRM for ATcase and high activity yet weak CRM for ATase. Six of the ten strains (SW3, 4, 5, 6, 9, and 10), which appeared to have different characteristics, such as shorter or longer half lives combined with strong or weak color formation

Table 4. Specific Activities of ATCase and ATase at $T_{2.5}$ and Color Formation From AP Immunoblotting

Strain	Specific activities (nmole/min/mg protein)		Color intensity	
	ATCase	ATase	ATCase	ATase
DB104	0.02	-	purple circles	
SW1	0.02	-	+++	+++
SW2	0.03	-	++	++
SW3	0.04	-	++	+++
SW5	0.03	-	+++	+
SW6	0.03	0.03	+++	+
SW7	0.01	-	+	+
SW8	-	0.04	++	+
SW9	0.06	0.24	++++	++++
SW10	0.77	0.11	+++	+++

-: undetectable level of enzyme activity
+, ++, +++, ++++: range of color intensity from the lightest to the darkest

(high or low CRM), were selected for further characterization in glucose starvation experiments.

During second glucose starvation experiments for the six strains plus DB104, 250 ml of cell cultures were harvested at 80 KU, 100 KU, and at 1.5 hours ($T_{1.5}$) and 3.0 hours ($T_{3.0}$) from the starting point of starvation. Again, the activities of ATCase and ATase were determined. The results were plotted as shown in Figures 4, 5, and 6. (Note: Only a part of the results of this experiment is presented here, because a third glucose starvation experiment, which was basically a repeat of this one, is presented later.) Activity of both enzymes, in general, increased during exponential growth and decreased upon starvation as shown in Figues 4 and 6. In some cases, such as SW3 (Figure 5), it is difficult to calculate the half life of enzyme activity accurately. The decrease of enzyme activity during starvation for several strains was different, which indicated different rates of inactivation. To clarify these results, a third glucose starvation experiment was required.

At this point in the project, DB104 began lysing at the end of exponential phase when grown on BMM, histidine and glucose medium. Numerous attempts were made to eliminate lysis, including alterations to the medium, changes in growth conditions of inoculum and the volume of starter cells. The only way to prevent DB104 from lysing was to include 20 amino acids (50 ug/ml each) in the medium. Therefore, from this point on, 20 amino acids were always added to BMM medium for growth of DB104.

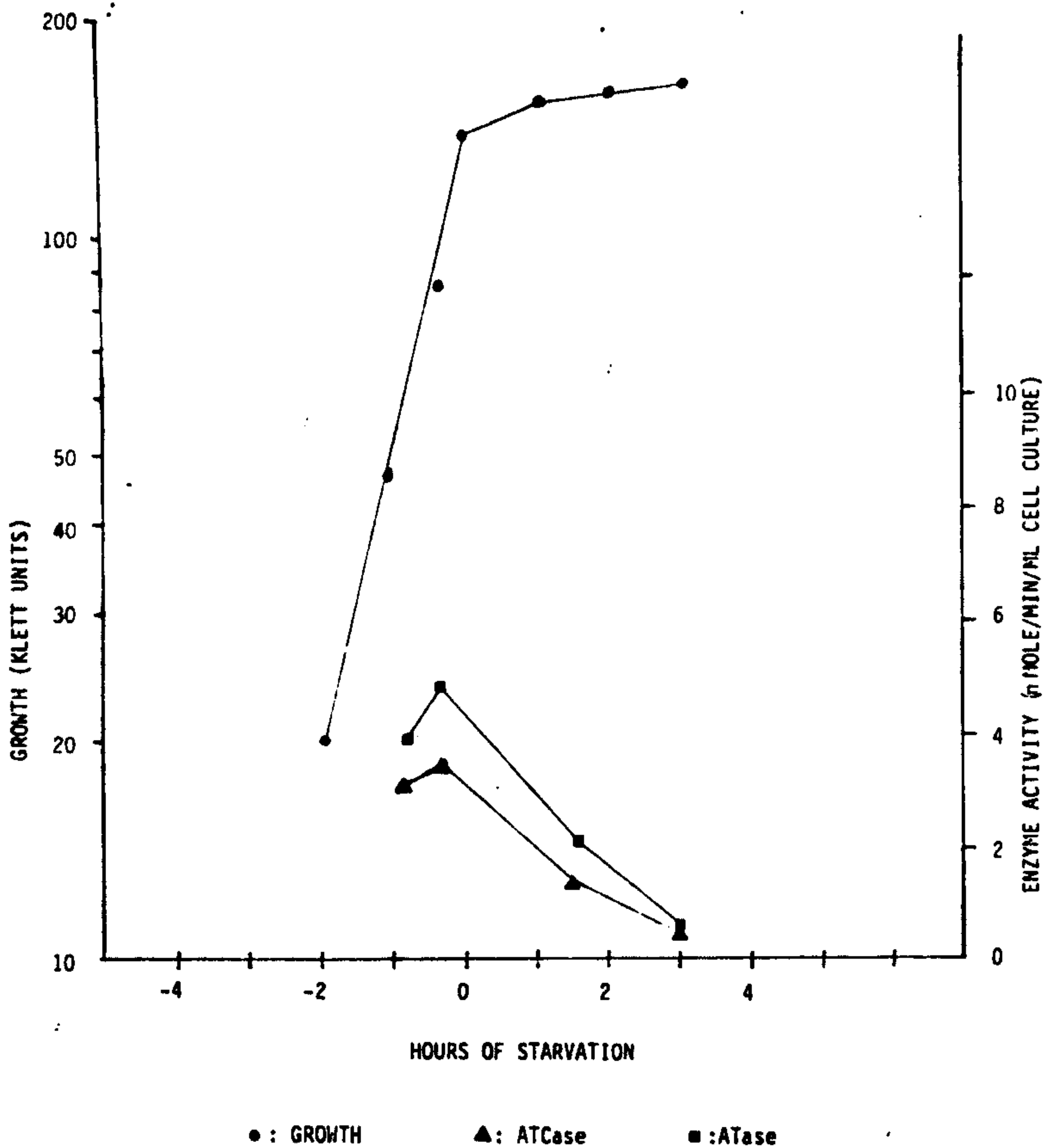

Figure 4. ATCase and ATase Activity in Strain DB104 During Glucose Starvation.

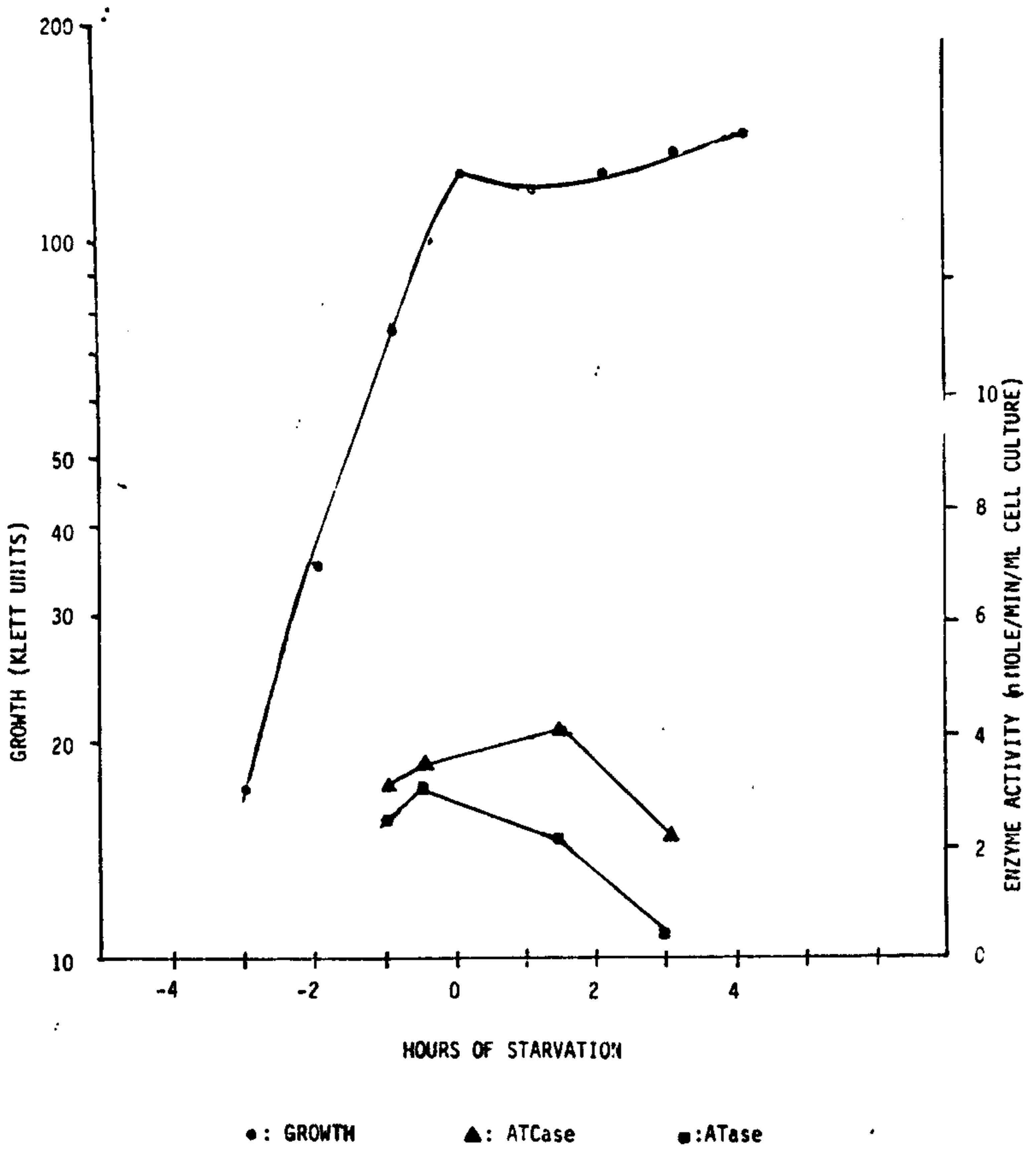

Figure 5. ATCase and ATase Activity in Strain SW3 During Glucose Starvation.

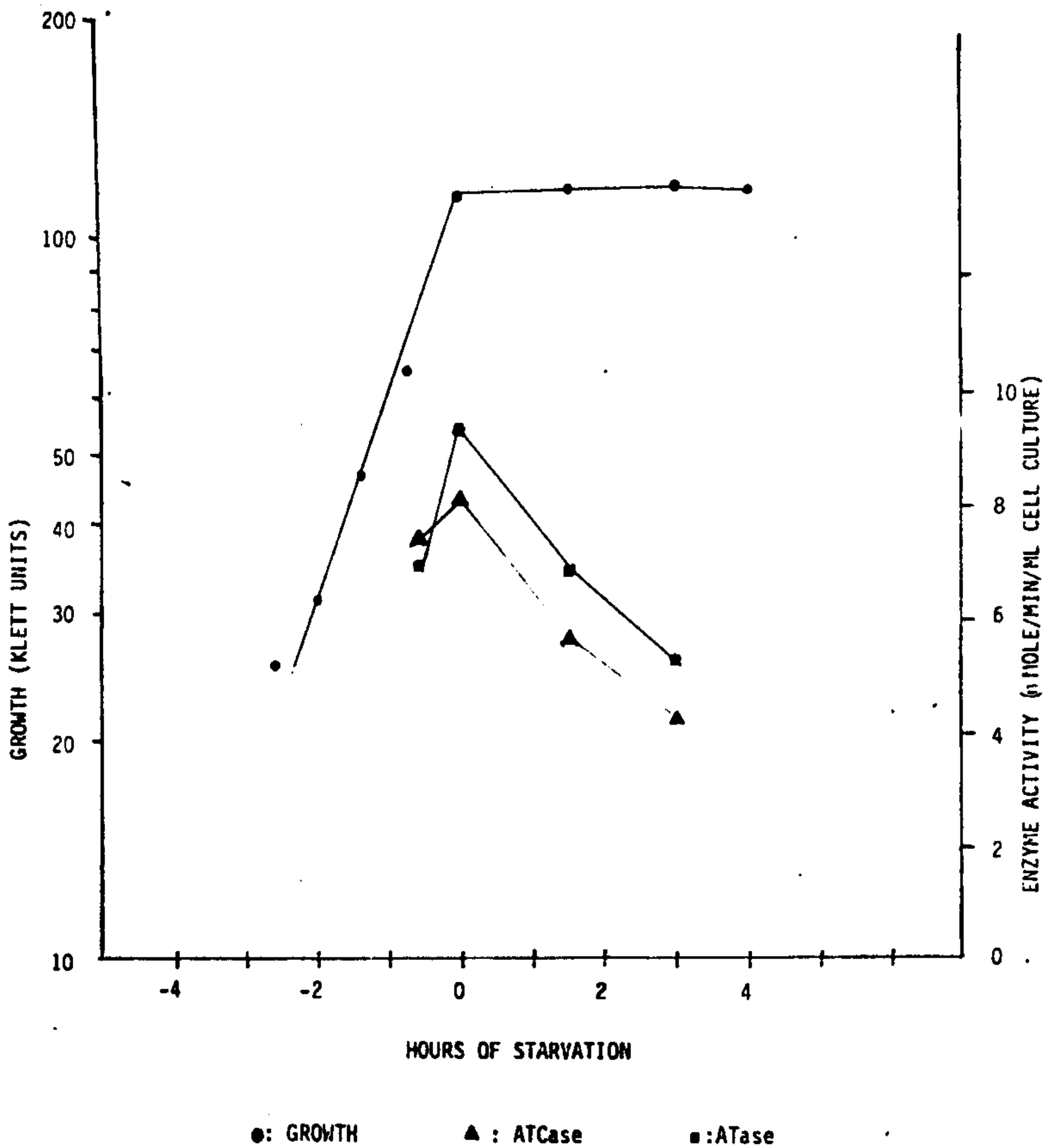

Figure 6. ATCase and ATase Activity in Strain SW9
During Glucose Starvation.

DB104 could still be glucose starved on BMM medium plus 20 amino acids. It should be noted that histidine was the only amino acid added to the mutant cell cultures and that the addition of 20 amino acids altered the doubling time of DB104 from 45 min to 25 min.

The third glucose starvation experiments were similar to the second, except that instead of harvesting 250 ml of the cell cultures, 150 ml samples were harvested at 80 KU, 100 KU, $T_{0.75hr}$, $T_{1.5hr}$, $T_{2.25hr}$, and $T_{3.5hr}$. The half lives of ATCase and ATase enzyme activity were calculated by determining the enzyme activity at each time point. The results are shown in Table 5. Comparing the half life of enzyme activity (Table 5) with the level of immunoblotting color (Table 4) leads to several conclusions. In most cases, long half life of enzyme activity corresponds to high levels of color. However, some strains have relatively short half lives of enzyme activity but high levels of color, such as ATase of SW3 and ATCase of SW4. This may reflect accumulation of inactive enzymes.

4) [^{3}H] Labeling and Immunoprecipatation

Immunoprecipatation is an essential procedure used to determine enzyme degradation. [^{3}H]leucine was added during early exponential growth. At T_0, when the cells started starving for glucose, nonradioactive leucine was added in excess to the cell culture to dilute out [^{3}H]leucine. Thus, proteins that were synthesized after T_0 should not incorporate the [^{3}H] label. The

Table 5. Half Lives of Enzyme Activities

	Half life in min	
Strain	ATCase	ATase
DB104*	90	90
SW3	90	60
SW4	75	100
SW5	>180	>180
SW6	>180	100
SW9	150	120
SW10	120	60

The half lives of enzyme activities were determined by measuring activities at T_0, $T_{0.75}$, $T_{1.5}$, $T_{2.25}$, and $T_{3.0}$.

* DB104 was grown on BMM plus 20 amino acids.

amount of ATCase and ATase protein present in cell extracts was estimated from the total amount of [3H] labeled cross reacting materials (CRM) found in immunoprecipitates after analysis by SDS polyacrylamide gel electrophoresis. The results of this experiment were plotted as % of the maximal cross reacting material (assuming maximal at T_0) vs. starvation time as shown in Figures 7 to 13. The growth curves and % of maximal enzyme activity are also ncluded. The level of CRM of both enzymes in wild type B. subtilis DB104 decreased at a rate corresponding to the rate of inactivation (Figures 7 and 8).

The loss of ATCase CRM during glucose starvation of strain SW9 was more rapid than the loss of ATCase activity (Figure 9). This result suggests that ATCase degradation is normal in SW9, but that ATCase synthesis continues during glucose starvation (unlike in wild type cells). Thus, ATCase activity appears to be lost more slowly than CRM because the effects of continued synthesis of ATCase are not detected in the pulse-chase design of the immunochemical experiments. SW9, which always had higher ATase activity than any other strain, also incorporated a higher amount of [^{3}H]leucine (at least 4 fold higher than DB104). The ATase CRM level in SW9 remained stable for over 3 hours of starvation, even though the enzyme activity half life was 90 min (Figure 10). These results suggest that SW9 overproduces ATase and is defective in degrada- tion of ATase. However, inactivation of this ensyme appeared normal.

The inactivation and degradation rates of ATCase and ATase in SW10 appeared to be parallel, but it should be noted that these rates were slower than in DB104. The results for SW3 and SW4 are not presented here because high background and irregular immunoprecipatation patterns were observed. But there were indications (from immunoblotting and enzyme assays) that SW4 accumulated inactive ATCase CRM. These results still need to be verified.

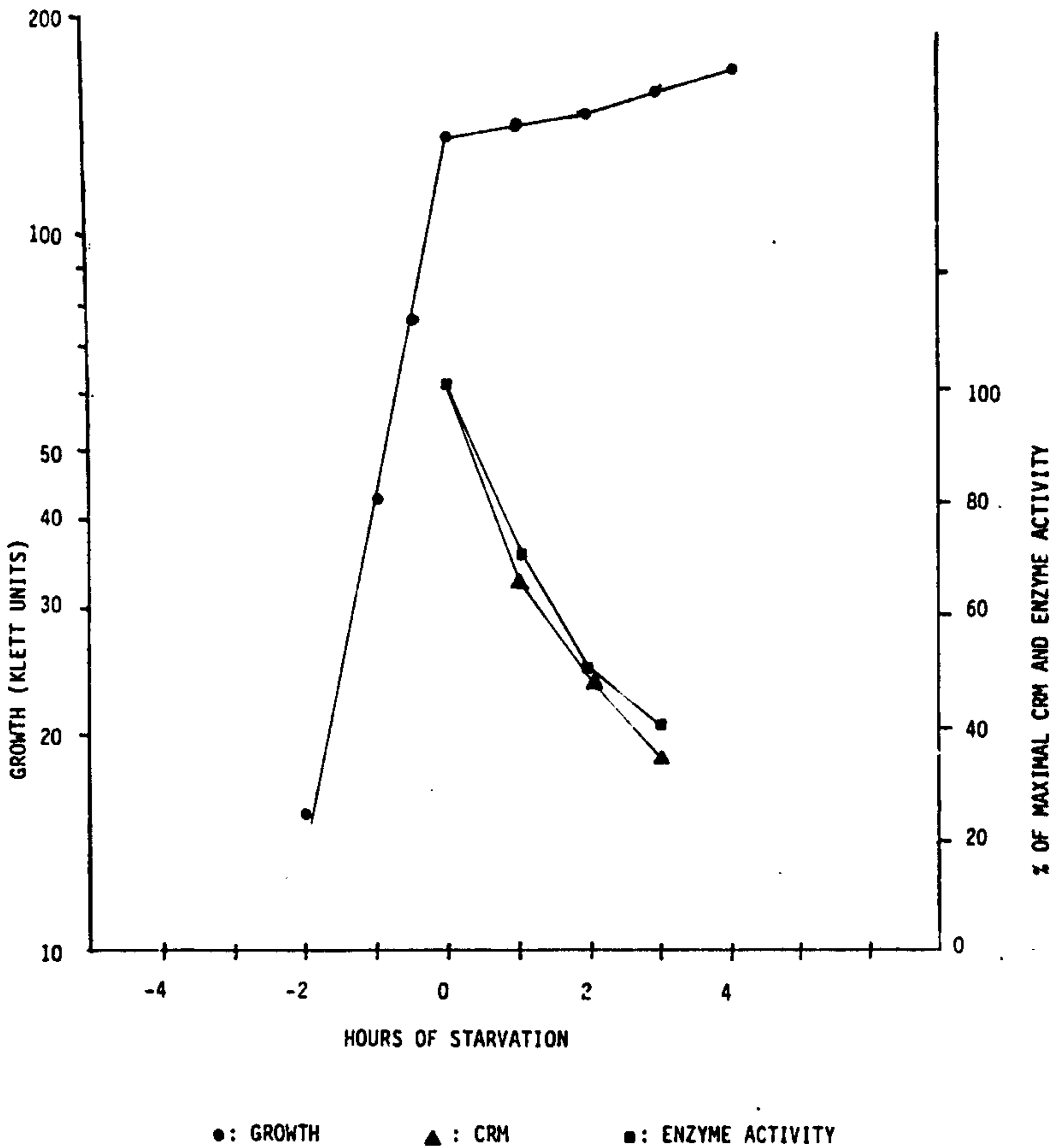

Figure 7. ATCase Activity and CRM in Strain DB104 During Glucose starvation.

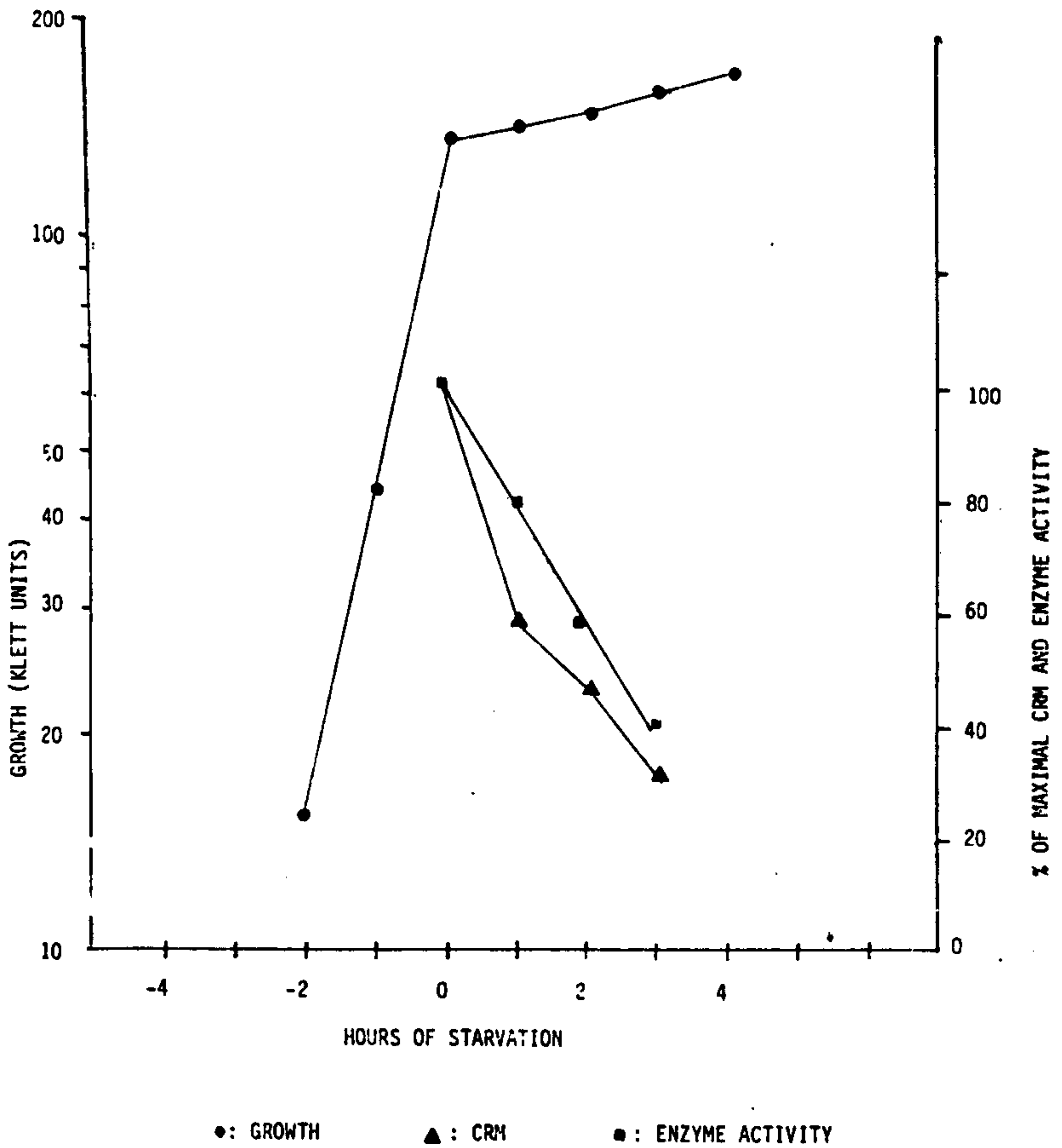

Figure 8. ATase Activity and CRM in Strain DB104 During Glucose Starvation.

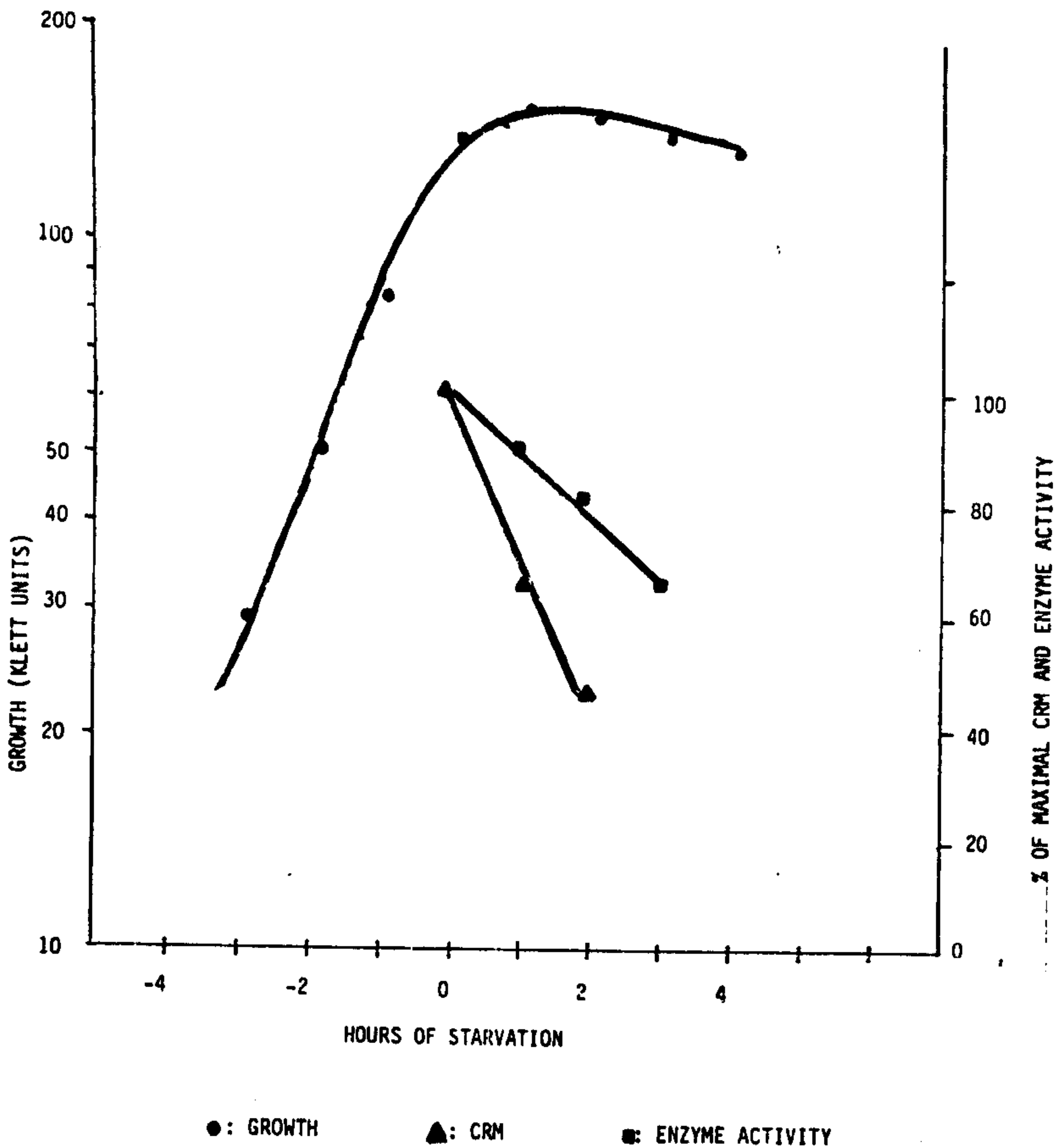

Figure 9. ATCase Activity and CRM in Strain SW9 During Glucose Starvation.

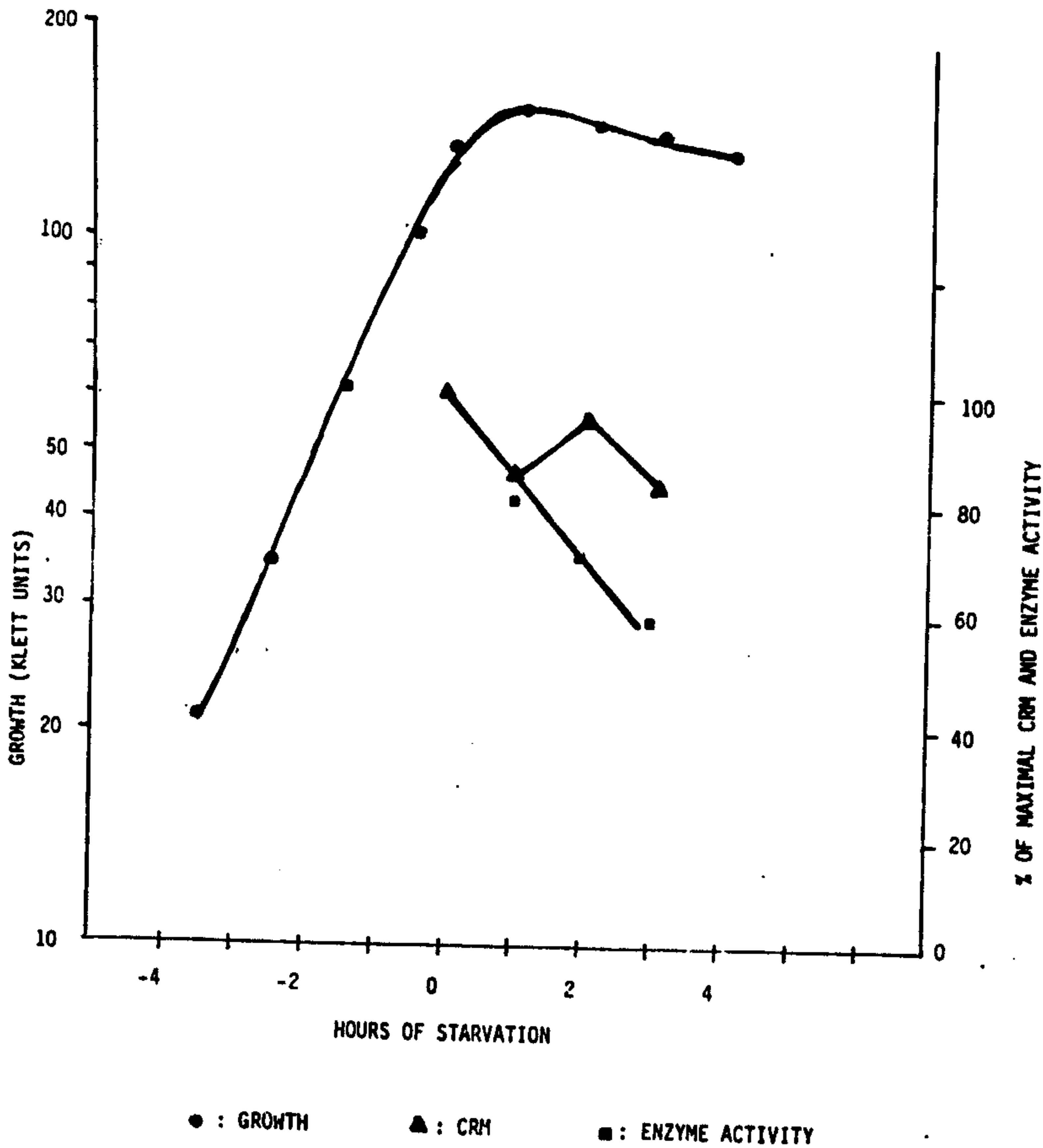

Figure 10. ATase Activity and CRM in Strain SW9 During Glucose Starvation.

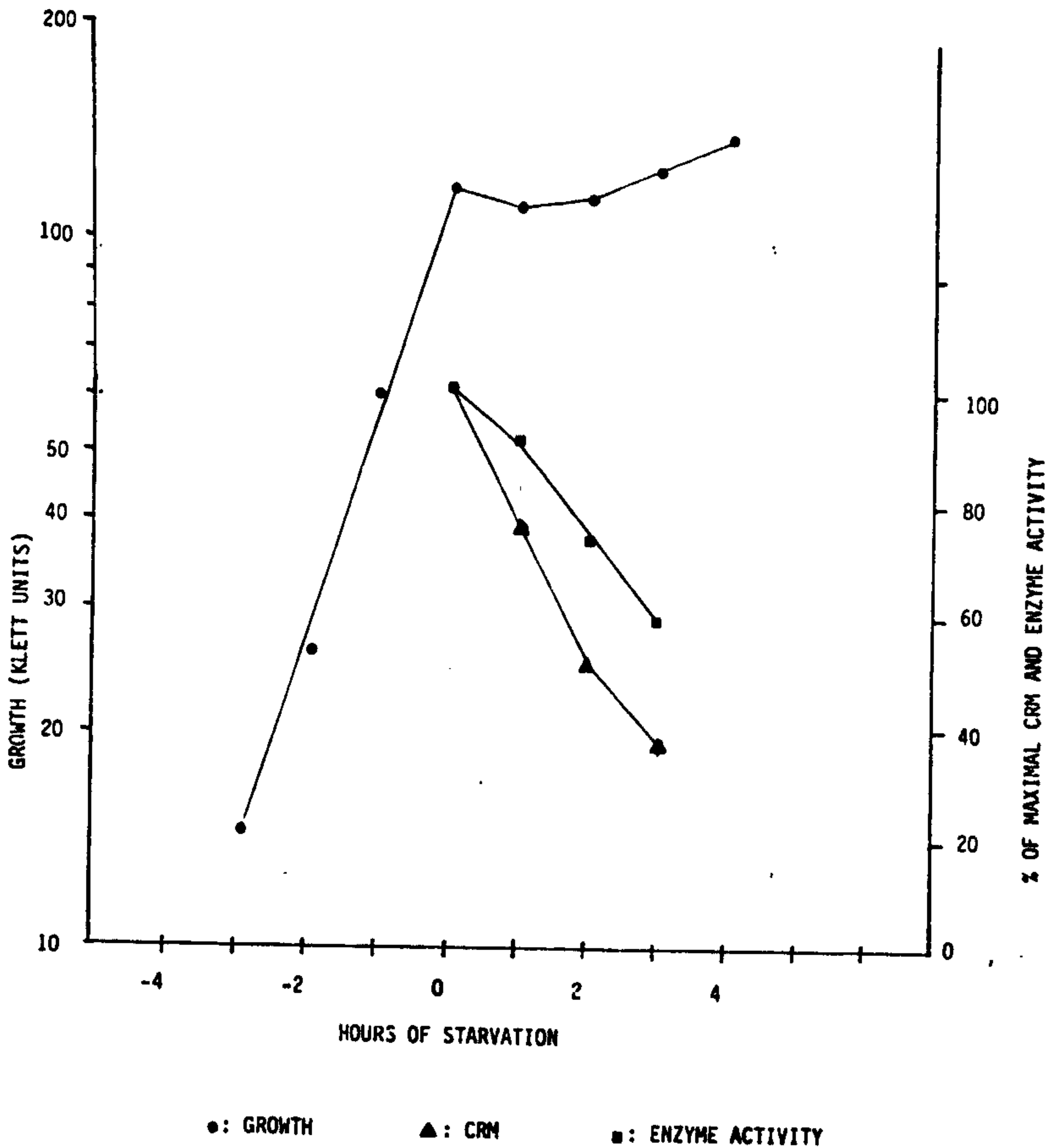

Figure 11. ATCase Activity and CRM in Strain SW10 During Glucose Starvation.

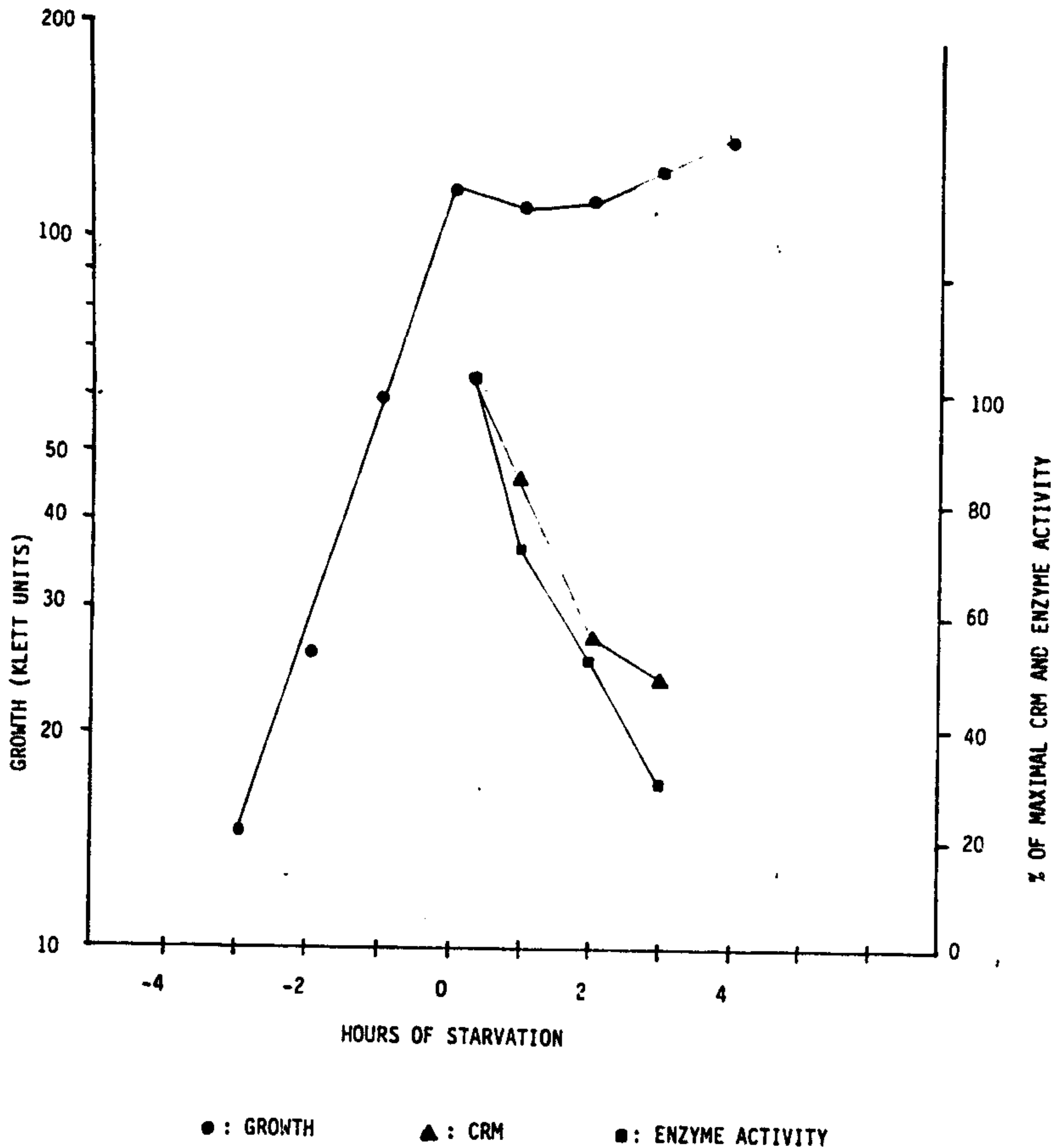

Figure 12. ATase Activity and CRM in Strain SW10 During Glucose Starvation.

DISCUSSION

The rationale of this system for isolation of mutants is the following. First, we assumed that whatever is involved in inactivation and degradation of ATCase and ATase can be altered by random mutation, and that this alteration will still allow normal cell growth. Second, it was assumed that the mutants that do not inactivate or degrade ATCase and/or ATase will outgrow wild type cells when they are transferred to fresh minimal medium lacking purines and pyrimidines (after a long preiod of starvation), since they do not need to resyrthesize these two enzymes. Third, when the cells are grown on minimal agar plates, the center cells of each colony will be starved earlier than the outer cells. So, for the wild type cells, the center cells will have little or none of these enzymes as compared to the outer cells. This is why we expected to see haloes after the staining procedure for wild type colonies and solid spots for mutants that maintain ATCase and/or ATase. The results suggest that our assumptions were correct.

DB104 was chosen as the parental strain for these studies, because it lacks two major extracellular proteases (alkaline and neutral protease). Thus, the possibility of degradation of ATCase and ATase by these two proteases can be eliminated.

For the purpose of this project, we wanted to collect a wide range of mutants. So we chose a mutagen, NTG, which has the ability of inducing many rare and multiple mutations, among which we hoped to find mutants that suit our interest. A number

of phenotypic classes would be expected to maintain stable levels of ATCase and/or ATase after a period of glucose starvation. These include strains that overproduce ATCase and/or ATase, _relA_ and _relC_ strains (Bond and Switzer, 1984 and Ruppen and Switzer, 1983), and other mutants that are defective in degrading either or both enzymes. The disadvantage of using NTG as a mutagen is that it is more difficult to perform genetic analysis on NTG mutants. This difficulty can be overcome in the future by using other less reactive chemical mutagens or the transposon Tn917 (Youngman _et al_., 1984) which induces single site mutations at a very low frequency.

The first problem we came across was optimizing the immunoblotting systems. The first immunoblotting kit used was the Protein A-HRP conjugate kit from Bio-Rad. The protein A-HRP conjugate turned out to be inactive. This was discovered when we were not able tc obtain any color reaction even when undiluted Protein A-HRP cojugate solution was added to color development solution. At that time, the manufacturer was unable to provide any active Protein A-HRP conjugate so, as a substitute, goat anti-rabbit IgG(H+L) HRP conjugate (which was supposed to be more sensitive) was used. Yet, none of the antibody dilutions that were suggested by the manufacturer worked. A significant amount of time was spent optimizing the concentrations of both primary and secondary antibodies. The concentrations chosen were 10 to 20 fold higher than suggested by Bio-Rad. Because of the highly unstable color formation with Bio-Rad system, we decided to try

another immunoblotting system, namely, the alkaline phosphatase conjugated goat anti-rabbit immunoscreening system sold by Promega.

During optimizing the immunoblotting systems, we also noticed that size of colonies at the time of lifting was critical for identification of positive clones. If the colonies were lifted while they were still very small in size, negative results could be easily mistaken for positive ones. The best results were obtained from plates containing 0.4% glucose, which had been incubated for about 36 to 40 hours.

Two sets of positive control strains were used in this project. E. coli strains harboring plasmids containing B. subtilis ATCase or ATase were used as controls. These strains were chosen because the levels of B. subtilis ATCase and ATase were significantly higher and more stable in E. coli than in B. subtilis. EB47 and EB48, which are E. coli strains containing low copy plasmids of B. subtilis ATCase and ATase, were first used but were later excluded, because they expressed very low levels of these enzymes. The level of ATase in EB48 was about 1/3 of wild type B. subtilis. This caused some confusion in interpreting the results because of poor color reaction with these strains. Therefore, TB2/pLS210 and TX158/pPZ2, which contain high copy number plasmids and produce easily detectable amounts of ATCase and ATase CRM, were used as controls.

The next problem was that the parental strain DB104, which grew normally during the first experiments of this project, began

lysing at the end of exponential growth when grown on BMM, histidine and glucose medium. This is in contrast to the mutants, which never lysed under the same condition. About 50% of the cells lysed within a half hour of starvation. Growth of the culture resumed after 2.5 hours. As mentioned previously, all 20 amino acids were included in later glucose starvation experiments to eliminate DB104 lysis. This change in growth conditions for DB104 made it a somewhat imperfect control. However, the real interest of this project was to obtain mutants that contained stable cross reacting material or showed stable enzyme activities during starvation. Thus, each strain could serve as its own control.

Several mutants have been isolated that express different phenotypes and appear to fall into our predicted mutant classes. In fact, from the data collected so far, SW9 which always produces high levels of ATase, (at least 2 fold higher than DB104) apparently does not degrade ATase (or if degradation occurs, it is extremely slow). One mutant (SW4) appears to be defective in degradation of ATCase but has a normal rate enzyme inactivation compared to DB104. This result needs to be confirmed, however. Some mutants are apparently defective in the inactivation of ATCase (SW5 and SW6) or ATase (SW5) during stationary phase (see Table 4). These observation also need to be confirmed, however.

Unfortunately, we were unable to completely characterize the mutants. However, this work has proven that our genetic approach to studying the complex system(s) of inactivation and degradation

of ATCase and ATase in *B. subtilis* is a useful one. Further work in this area will include further charaterization of the current mutants, as well as use of new mutagenic procedures. The results will hopefully help to elucidate the specific components of this developmental system.

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